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Impact of p53 function on the sulfotransferase-mediated bioactivation of the alkylated polycyclic aromatic hydrocarbon 1-hydroxymethylpyrene *in vitro*

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ABSTRACT

The tumor suppressor p53, encoded by *TP53*, is known as the 'guardian of the genome'. Sulfotransferases (SULTs) are involved in the metabolism of alkylated polycyclic aromatic hydrocarbons such 1-hydroxymethylpyrene (1-HMP) which is a known substrate for SULT1A1. To investigate the impact of *TP53* on the metabolic activation of 1-HMP a panel of isogenic human colorectal HCT116 cells having *TP53(+/+)*, *TP53(+/-)* or *TP53(-/-)* were treated with 10 μM 1-HMP for 24 hours. 1-HMP-DNA adduct formation was determined by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis, which quantified two nucleoside adducts N^2-(1-methylpyrenyl)-2'-deoxyguanosine (MPdG) and N^6-(1-methylpyrenyl)-2'-deoxyadenosine (MPdA). 1-HMP treatment resulted in significantly (~40-fold) higher DNA adduct levels in *TP53(+/+)* cells than in the other cell lines. Higher levels of 1-HMP-induced DNA adducts in *TP53(+/+)* cells correlated with higher basal expression of SULT1A1/3 in this cell line but 1-HMP treatment showed no effect on the expression of this protein. These results indicate that the cellular *TP53* status is linked to the SULT1A1/3-mediated bioactivation of 1-HMP thereby broadening the spectrum of p53's targets.

Key words: tumor suppressor p53, carcinogen activation, sulfotransferase, DNA adduct, alkylated polycyclic aromatic hydrocarbons

INTRODUCTION

Like unsubstituted polycyclic aromatic hydrocarbons (PAHs), alkylated PAHs are also formed through incomplete combustion of organic matter, although preferentially at lower combustion temperatures (Flesher and Lehner 2016). They can also be generated as a result of diagenic processes of organic sediments (e.g. mineral oil and coal) (Richter-Brockmann and Achten 2018). 1-Methylpyrene (1-MP), one such alkylated PAH, is a widespread environmental pollutant detectable in various foods (native olive oil, smoked cheese, cooking margarine, coffee brew, and grilled sausages) and it bioaccumulates in marine creatures such as mussels, crabs and finfish (Bendadani et al. 2014a; Bendadani et al. 2014b). It can also be found in car exhausts and is present in cigarette smoke condensate at higher concentrations than the reference PAH, benzo[*a*]pyrene (BaP) (Bakhiya et al. 2006). 1-MP is hepatocarcinogenic in rodents (Rice et al. 1987) but to date insufficient data are available on the carcinogenic risk to humans. Alkylated PAHs like 1-MP require metabolic activation into electrophilic intermediates to exert their genotoxic effects (Flesher and Lehner 2016). CYP-catalyzed biotransformation of 1-MP yields 1-hydroxymethylpyrene (1-HMP), which is further activated by sulfotransferases (SULTs), particularly SULT1A1, to 1-sulfoxymethylpyrene (1-SMP) (**Figure 1**) (Bendadani et al. 2014a). 1-SMP forms DNA adducts *in vitro* and *in vivo*, with N^2-(1-methylpyrenyl-2'-deoxyguanosine (MPdG) and N^6-(1-methylpyrenyl)-2'-deoxyadenosine (MPdA) identified as major adducts (Monien et al. 2008). 1-SMP has been shown to induce tumors in mice and rats (Surh et al. 1990; Horn et al. 1996).

The tumor suppressor p53, encoded by *TP53*, is mutated in approximately 50% of sporadic tumors making it arguably one of the most important cancer genes (Olivier et al. 2010). p53 can be induced by multiple forms of cellular stress (e.g. DNA damage) and it functions at the crossroads of a network of signaling pathways that are crucial for regulating cell growth and apoptosis. Its response to DNA damage is to abrogate DNA synthesis or cell division and this has earned p53 the term 'guardian of the genome'. Disruption of the normal p53 response by *TP53* mutation leads to the development of tumors and various carcinogens have been associated with characteristic mutational signatures (Kucab et al. 2015). In addition to the role of p53 in DNA damage response, new functions are still being discovered. Over recent years a role for p53 in modulating carcinogen metabolism has emerged (Hockley et al. 2008; Simoes et al. 2008; Krais et al. 2016a; Krais et al. 2016b; Wohak et al. 2016; Willis et al. 2018; Wohak et al. 2018). We have shown that DNA adduct formation by BaP was significantly impacted by p53 function both *in vitro* and *in vivo* and that the observed DNA damage correlates with CYP1A1 expression (Krais et al. 2016a; Wohak et al. 2016). A recent

study of the air pollutant 3-nitrobenzanthrone (3-NBA) and its metabolites 3-aminobenzanthrone (3-ABA) and *N*-hydroxy-3-aminobenzanthrone (*N*-OH-3-ABA) illustrated that p53's influence on carcinogen metabolism depends on the class of carcinogen and on the xenobiotic-metabolizing enzymes (XMEs) mediating the bioactivation (Wohak et al. 2018). This was also the first study indicating that p53 can impact on SULT-mediated (i.e. SULT1A1) carcinogen metabolism (Wohak et al. 2018).

In the present study we used isogenic human colorectal HCT116 cells differing in their *TP53* status as model to further investigate the impact of cellular p53 function on SULT-mediated carcinogen activation. HCT116 *TP53(+/+)*, *TP53(+/-)*, and *TP53(-/-)* cells were compared for their ability to metabolically activate 1-HMP. 1-HMP-DNA adduct formation was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and SULT1A1/3 expression was determined by Western blotting.

MATERIAL AND METHODS

Carcinogen

1-HMP (CAS no. 24463-15-9; purity 98%) was purchased from Sigma Aldrich.

Cell culture and treatment

Human colorectal HCT116 *TP53(+/+)*, *TP53(+/-)*, and *TP53(-/-)* cells were kindly provided by Prof. Bert Vogelstein, Johns Hopkins University School of Medicine, Baltimore, MD. Cells were grown as adherent monolayers as described (Wohak et al. 2016; Wohak et al. 2018). For treatment, cells were seeded at 3×10^4 cells/cm^2, grown for 48 hours and subsequently treated for up to 48 hours with 0, 1, 10, or 20 µM 1-HMP dissolved in dimethyl sulfoxide (DMSO; 0.5% final volume of medium).

Cell viability

Cells were seeded on 96-well plates for up to 48 hours. Cell viability after 1-HMP treatment was assessed using a crystal violet staining assay as reported (Wohak et al. 2018).

DNA adduct analysis

Cells were seeded in 75-cm^2 flasks and treated with 10 µM 1-HMP for 24 hours. To obtain sufficient DNA (~100 µg) for analysis cells were pooled from two flasks prior DNA isolation using a standard phenol/chloroform extraction method. 1-HMP-DNA adducts were measured by isotope-dilution ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) as described elsewhere (Monien et al. 2008). Briefly, DNA (100 µg) was spiked with isotope-labeled standards (250 fmol [$^{15}N_5$]MPdA and 2.5 pmol [$^{15}N_5$,$^{13}C_{10}$]MPdG), digested enzymatically to nucleosides, and subjected to solid-phase extraction for enrichment of the adducts as reported (Monien et al. 2008; Monien et al. 2009). Sample separation was performed using an Acquity ultraperformance liquid chromatography (UPLC) system with a BEH Phenyl column (1.7 µm, 2.1 × 100 mm) and analyzed with a Quattro Premier XE tandem quadrupole mass spectrometer using an electrospray interface operated in the positive ion mode (all from Waters, Eschborn, Germany). For quantification (quantifier) the neutral loss of the furanose unit (MPdA 466.3 → 350.1 and MPdG 482.3 → 366.1) was used with [$^{15}N_5$]MPdA (471.3 → 355.1) and [$^{15}N_5$ $^{13}C_{10}$]MPdG (497.3 → 376.1) as internal standards, while the fragmentation into nucleoside and the 1-MP cation (*m/z* = 215.1)

was used to confirm the identity of the substance (qualifier). The tune parameters for MPdA were [deviating parameters for MPdG are given in parentheses]: temperature of the electrospray source, 110°C; desolvation temperature, 485°C [MPdG 495°C]; desolvation gas, nitrogen (850 L/hour); cone gas, nitrogen (50 L/hour); collision gas, argon (indicated cell pressure ~4.6×10^{-3} mbar). Fragmentation of MPdA required 35 and 18 eV collision energies for the transitions 466.3 → 215.1 and 466.3 → 350.3, respectively. Collision energies for fragmentation of MPdG were 30 and 10 eV for the transitions 482.3 → 215.1 and 482.3 → 366.3, respectively. Further experimental parameters were set as follows: dwell time, 100 ms; capillary voltage, 4 kV (MPdG 3.8 kV); cone voltage, 30 (MPdG 25 V); RF1 lens voltage, 0.1 V. Data acquisition and handling were performed with MassLynx software. The limits of detection (LOD) were at approximately 3 MPdG and 0.6 MPdA per 10^8 nucleosides (Monien et al. 2008). All samples were analyzed twice, and results were usually within 15% variation.

Western blot analysis

Cells were seeded in 25-cm^2 flasks and treated with 10 µM 1-HMP for up to 48 hours. Western blotting was performed as described (Wohak et al. 2018). Primary antibodies and dilutions used were: anti-p53 1:2000 (Ab-6; Calbiochem), anti-p21 (CDKN1A) 1:2000 (556431; BD Pharmingen) and anti-SULT1A1/3 1:5000. Anti-SULT1A1/3 was a generous gift from Prof. Hansruedi Glatt (German Institute of Human Nutrition, Nuthetal, Germany). This antiserum was raised in rabbits against bacterial inclusion bodies of human SULT1A (Teubner et al. 2007) and was shown to exhibit some cross-reactivity detecting human SULT1A1 and SULT1A3. β-Actin (1:20000; ab6276, Abcam) was used as loading control.

Statistical analysis

Statistical analyses were performed with Prism GraphPad Software (Version 7.04) and $p < 0.05$ was considered significant.

RESULTS

Cell viability in HCT116 cells after exposure to 1-HMP

Firstly, the effect of 1-HMP on the viability of HCT116 cells was evaluated (**Figure 2**). *TP53(+/+)*, *TP53(+/-)*, and *TP53(-/-)* cells were exposed to 5, 10 and 20 μM 1-HMP or DMSO alone (control) for 24 and 48 hours. These concentrations were chosen according to previous studies (Glatt et al. 1994; Jiang et al. 2015). *TP53(+/-)* cells showed the strongest sensitivity to 1-HMP exposure; at 20 μM viability was decreased to 77% and 72% after 24 and 48 hours, respectively (**Figure 2**). At the lower concentrations of 5 and 10 μM *TP53(+/-)* cell viability was >85%. For *TP53(+/+)* and *TP53(-/-)* cells at least 85% stayed viable at all the concentrations and time points tested (**Figure 2**).

DNA adduct formation in HCT116 cells treated with 1-HMP

Based on some initial tests in HCT116 *TP53(+/+)* cells (data not shown), 10 μM 1-HMP and a 24-hour incubation time were selected to compare 1-HMP-induced DNA adduct formation in *TP53(+/+)*, *TP53(+/-)* and *TP53(-/-)* cells. **Figure 3B** shows representative UPLC-MS/MS chromatograms of digested DNA from 1-HMP-treated *TP53(+/+)* cells. MPdG levels were about 15-fold higher than MPdA levels in all cell lines (**Figure 3A**). This trend coincides with previous findings that reported MPdG to be the predominant 1-HMP-DNA adduct formed (Bendadani et al. 2014b). Strikingly, levels of both DNA adducts were significantly higher in *TP53(+/+)* cells than in *TP53(+/-)* and *TP53(-/-)* cells (**Figure 3A**) (MPdA: ~40-fold compared to *TP53(+/-)* and *TP53(-/-)* cells; MPdG: ~24-fold compared to *TP53(+/-)* cells, ~13-fold compared to *TP53(-/-)* cells).

Impact of TP53 status on the expression of DNA damage response proteins and SULTs in HCT116 cells treated with 1-HMP

For Western blot analysis *TP53(+/-)*, *TP53(+/-)*, and *TP53(-/-)* cells were treated with 10 μM 1-HMP for 24 and 48 hours and whole cell lysates were analyzed for the expression of p53, p21 and SULT1A1/3. 1-HMP caused a strong induction of p53 and its downstream target p21 in *TP53(+/+)* cells (**Figure 4**). *TP53(+/-)* cells exhibited very low expression and no induction of p53 and p21 under these experimental conditions, whereas p53 was not detectable in *TP53(-/-)* cells. Basal SULT1A1/3 protein expression was much higher in *TP53(+/+)* cells than in *TP53(-/-)* and *TP53(+/-)* cells, which correlates with the significantly higher 1-HMP-

DNA adduct levels in the former. Treatment with 1-HMP had no impact on SULT1A1/3 expression in any of the cell lines.

DISCUSSION

Previous studies in HCT116 cells have investigated the impact of p53 on the metabolic activation of several PAHs (e.g. BaP, dibenz[*a,h*]anthracene [DBA], dibenzo[*a,l*]pyrene [DBP]) and some nitroarenes (e.g. 3-NBA and aristolochic acid I) (Hockley et al. 2008; Simoes et al. 2008; Wohak et al. 2016; Wohak et al. 2018). A study of 3-NBA and its metabolites 3-ABA and *N*-OH-3-ABA included investigations of SULTs, because the genotoxicity of 3-NBA is strongly enhanced by SULT1A1 and SULT1A2 (Arlt et al. 2017). Although treatment with 3-NBA, 3-ABA, or *N*-OH-3-ABA had no effect on the induction of SULT1A1/3 proteins, results revealed a clear impact of *TP53* status on basal expression of these enzymes (Wohak et al. 2018). To examine this phenomenon further 1-HMP was chosen in the present study as it is bioactivated mainly by SULTs (i.e. SULT1A1).

1-HMP-DNA adduct analysis in HCT116 cells revealed clearly the impact of *TP53* genotype on the SULT-mediated bioactivation of this compound, seen in higher MPdA and MPdG adduct levels in *TP53*(+/+) cells compared with *TP53*(+/-) and *TP53*(-/-) cells. MPdG levels were found to be ~20-fold higher than MPdA levels, in agreement with previous findings (Bendadani et al. 2014a; Bendadani et al. 2014b). The significantly higher adduct levels in *TP53*(+/+) cells could be explained by correspondingly higher SULT1A1/3 levels in this cell line, than in *TP53*(+/-) and *TP53*(-/-) cells.

Bioactivation of 1-HMP in humans can be catalyzed by several SULTs, of which human SULT1A1 is reported to be by far the most efficient followed by 1E1. Furthermore SULT1A2, 1A3, 1B1, 1C2, 1C3, 2A1, and 2E1 also convert 1-HMP into a genotoxic metabolite, but to much lower extents (Meinl et al. 2002; Meinl et al. 2013; Jiang et al. 2015). The antibody raised against SULT1A used in the present study detects SULT1A1 and 1A3 with a similar affinity. Both SULTs are prevalent in human colon (Dobbernack et al. 2011) although SULT1A1 expression has been demonstrated to be ~2-fold lower than SULT1A3 (Teubner et al. 2007). In human intestinal carcinoma Caco-2 cells the difference is ~15-fold (Meinl et al. 2008). Besides SULT1A1 five other SULTs were found in these cells (SULT1A2, 1B1, 1C2, 2A1, 1E1) with 1E1 being expressed only at very low levels. We can speculate that because SULT1A1 is much more efficient than SULT1A3 in activating 1-HMP (~110-fold) (Meinl et al. 2002; Meinl et al. 2013) the demonstrated impact of p53 on the bioactivation of 1-HMP in HCT116 cells is likely through SULT1A1 and not SULT1A3. In accordance with our study, Rothman and coworkers also found substantial higher basal SULT1A1 protein levels in HCT116 *TP53*(+/+) cells compared with *TP53*(-/-) cells, but they did not investigate SULT1A3

(Rothman et al. 2015). Other SULTs in HCT116 cells have not been investigated, but should be considered when testing other carcinogens. The only other SULT detected in human colon that has been shown to bioactivate 1-HMP is SULT1B1 (Teubner et al. 2007).

An implication of the effect of p53 on SULT expression is that it could affect the toxicity of some dietary carcinogens and other environmental pollutants. A previous study has already indicated the tissue-dependent impact of p53 on the biotransformation of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Krais et al. 2016b). In addition to Cyp1a enzymes, Sult1a1 contributed to PhIP-DNA adduct formation in *Trp53(+/+)*, *Trp53(+/-)*, and *Trp53(-/-)* mice. Other examples that have yet to be studied are furfuryl alcohol and 5-hydroxymethylfurfural, rodent carcinogens that are activated to reactive intermediates by SULT1A1 (Sachse et al. 2014; Sachse et al. 2016). As mentioned, SULTs are also involved in the bioactivation of several environmental pollutants that carry nitro- or amino groups; for example, carcinogenic nitro- and aminotoluenes (Glatt et al. 2016). Used in large quantities for the synthesis of dyes, pesticides and synthetic rubbers, they may represent an occupational exposure hazard. Aminotoluenes also occur in tobacco smoke but the potential impact of p53 on their SULT-mediated biotransformation remains to be investigated.

In HCT116 cells, p53-mediated induction of CYP1A1 was observed after treatment with PAHs such as BaP, DBA and DBP (Wohak et al. 2016). When studying several chemotherapeutic drugs, it was found that etoposide and ellipticine induced CYP1A1 in HCT116 *TP53(+/+)* cells, but not in HCT116 *TP53(-/-)*, demonstrating that the mechanism of CYP1A1 induction was p53-dependent, whereas cisplatin had no such effects (Willis et al. 2018). In the present study, 1-HMP treatment did not result in the induction of SULT1A1/3. To date transcriptional regulation of SULTs is not well understood and a broad spectrum of transcription factors has been indicated to play a role in the regulation of these enzymes, e.g. PXR and CAR (Runge-Morris et al. 2013). Thus, further investigations are needed to clarify how p53 impacts on the basal expression of SULT1A in HCT116 cells.

Conclusions

This study clearly links cellular *TP53* status to the SULT1A1/3-mediated bioactivation of 1-HMP *in vitro* thereby broadening the spectrum of p53's targets. This could be a critical determinant of genotoxicity as numerous environmental and dietary carcinogens are activated to chemically reactive metabolites via sulfation (O-sulfonation). As the underlying mechanism by which p53 function influences the expression of SULT1A1 remain elusive, we acknowledge that the effects observed in 1-HMF-treated HCT116 cells should be confirmed in other isogenic human cells that have been created using a different *TP53* knockout strategy (e.g. CRISPR/Cas9 technology). Future investigations should include both *in vitro* and *in vivo* models to examine the impact of p53 on SULT-mediated bioactivation of 1-HMP. Since SULT1A1/3 plays a role in maintaining homeostasis of steroids and thyroid hormones these observations could also link p53 to other physiological pathways. Our results again highlight that gene-environment interactions need to be considered when studying carcinogen metabolism.

AUTHOR CONTRIBUTIONS

LEW and VMA initiated the study, had the main responsibility for the study design, interpretation and for finalizing the manuscript. LEW conducted all the cell culture work. BM conducted the DNA adduct analysis. DHP was involved in the study design, interpretation and contributed to the writing of the manuscript. All authors critically reviewed the manuscript and approved it.

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FIGURE LEGENDS

Figure 1: Bioactivation and DNA adduct formation of 1-hydroxymethylpyrene (1-HMP). 1-HMP is converted by sulfotransferase 1A1 (SULT1A1) to the reactive intermediate 1-sulfoxymethylpyrene (1-SMP) and the DNA adducts N^6-(1-methylpyrenyl)-2'-deoxyadenosine (MPdA) and N^2-(1-methylpyrenyl)-2'-deoxyguanosine (MPdG).

Figure 2: Effects of 1-HMP on cell viability in HCT116 *TP53(+/+)*, *TP53(+/-)*, and *TP53(-/-)* cells treated with 1-HMP for 24 (A) and 48 hours (B). Values represent the mean ± SD (*n*=4); two separate incubations with two biological samples each.

Figure 3: (A) 1-HMP-DNA adduct levels in HCT116 *TP53(+/+)*, *TP53(+/-)*, and *TP53(-/-)* cells treated with 10 µM 1-HMP for 24 hours. MPdA and MPdG adducts were quantified by LC-MS/MS. Values represent the mean ± SD (*n*=4); two separate incubations with two biological samples each. Statistical analysis was performed by one-way ANOVA followed by the Tukey post-hoc test (***p*<0.01, ****p*<0.005, different from HCT116 *TP53(+/+)* cells). Please note different scales in panels for MPdA and MPdG adducts. (B) UPLC-MS/MS chromatograms of digested DNA from HCT116 *TP53(+/+)* cells following incubation with 10 µM 1-HMP for 24 hours. Results shown are representative of those obtained in 1-HMF-exposed HCT116 *TP53(+/-)* and *TP53(-/-)* cells. Fragmentations of the analytes N^6-MPdA, *m/z* = 466 → 350 (first panel), and N^2-MPdG, *m/z* = 482 → 366 (third panel), were monitored together with the transitions of the internal standard substances [$^{15}N_5$]N^6-MPdA, *m/z* = 471 → 355 (second panel), and [$^{13}C_{10}$,$^{15}N_5$]N^2-MPdG, *m/z* = 497 → 376 (fourth panel). The ratio of the peak areas for the transition *m/z* = 466 → 350 (N^6-MPdA) and for *m/z* = 471 → 355 ([$^{15}N_5$]N^6-MPdA) was used to calculate the N^6-MPdA content of the DNA. Accordingly, the N^2-MPdG was determined from the peak areas of *m/z* = 482 → 366 (N^2-MPdG) and *m/z* = 497 → 376 ([$^{13}C_{10}$,$^{15}N_5$]N^2-MPdG).

Figure 4: Protein expression of p53, p21, and SULT1A1/3 in HCT116 *TP53(+/+)*, *TP53(+/-)*, and *TP53(-/-)* cells treated with 10 µM 1-HMP for 24 and 48 hours. Blots shown are representative of experiments conducted at least twice and β-actin was used as loading control.

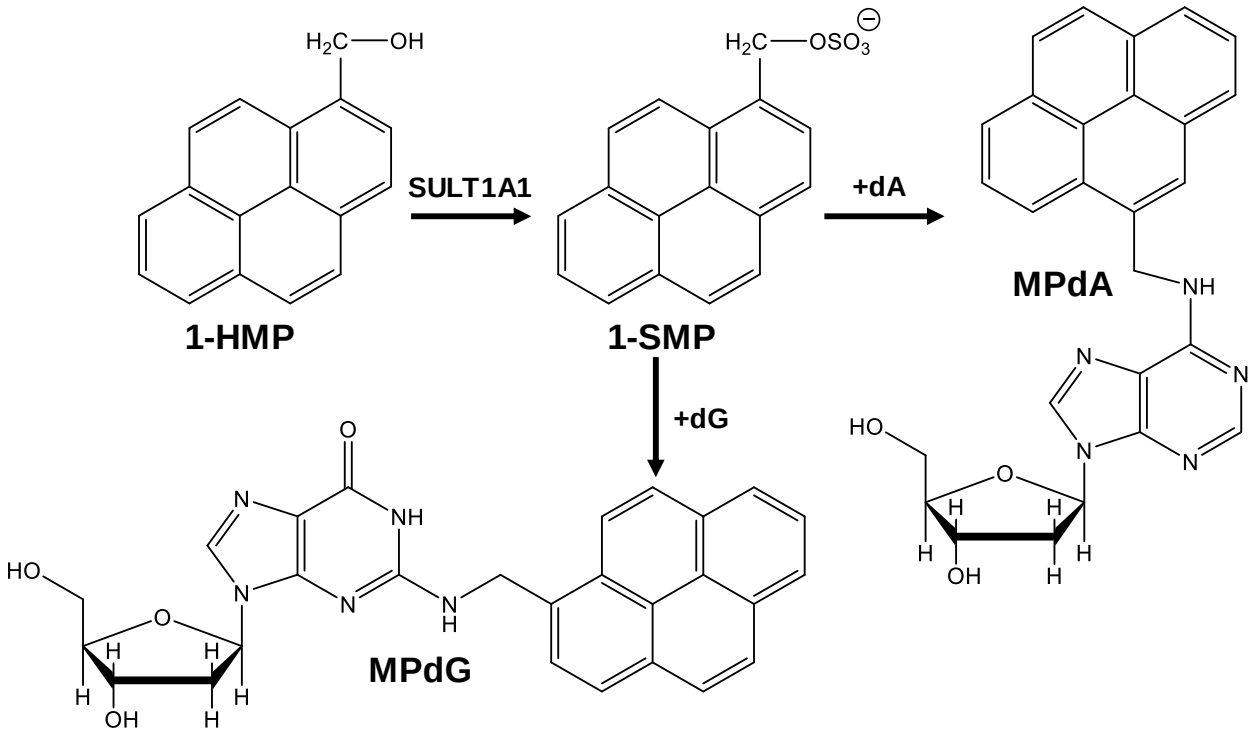

Figure 1

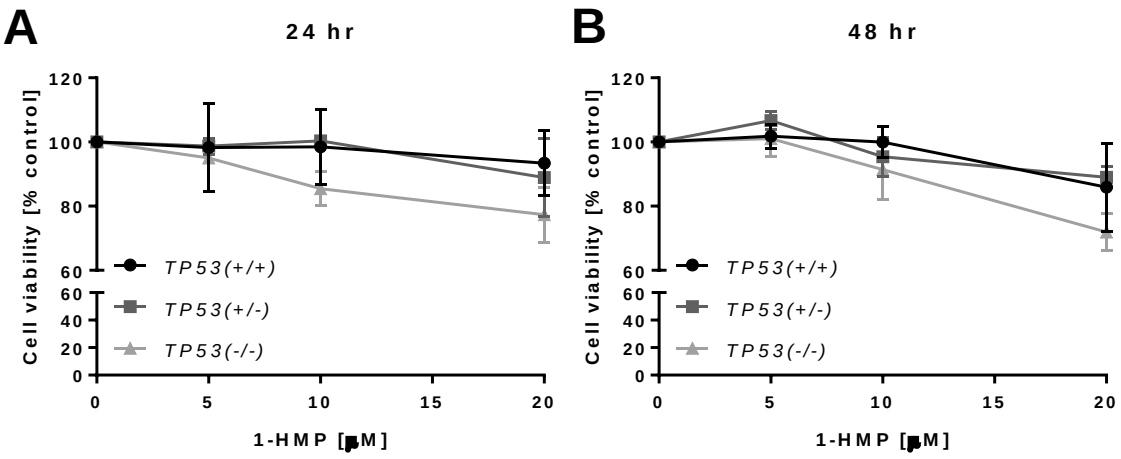

Figure 2

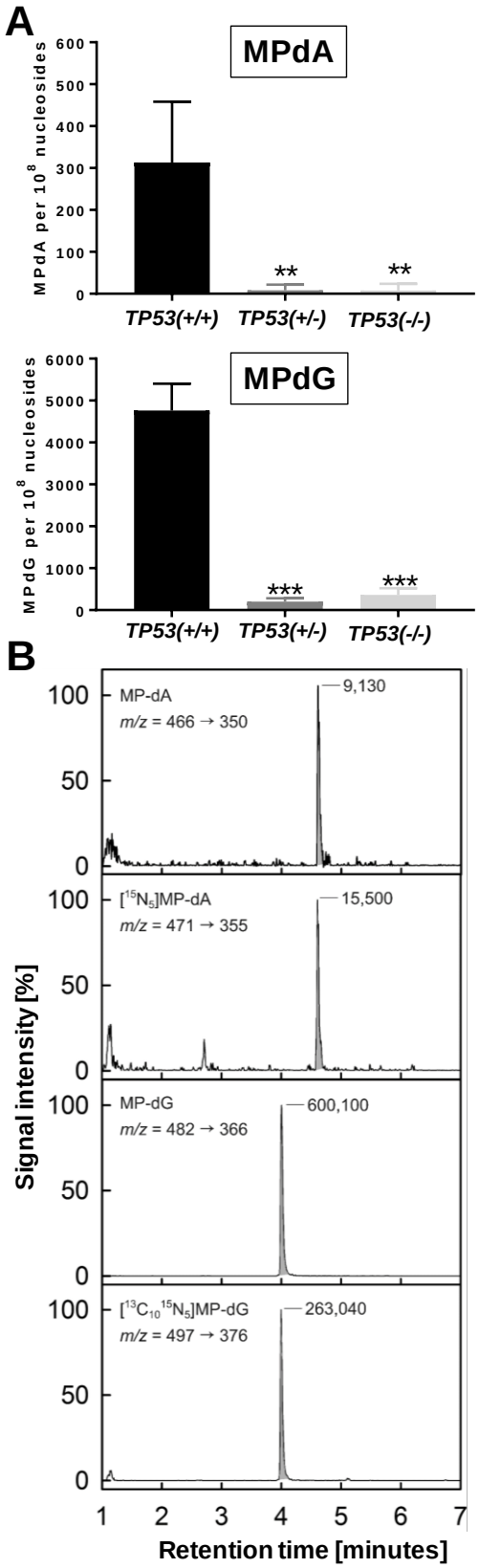

Figure 3

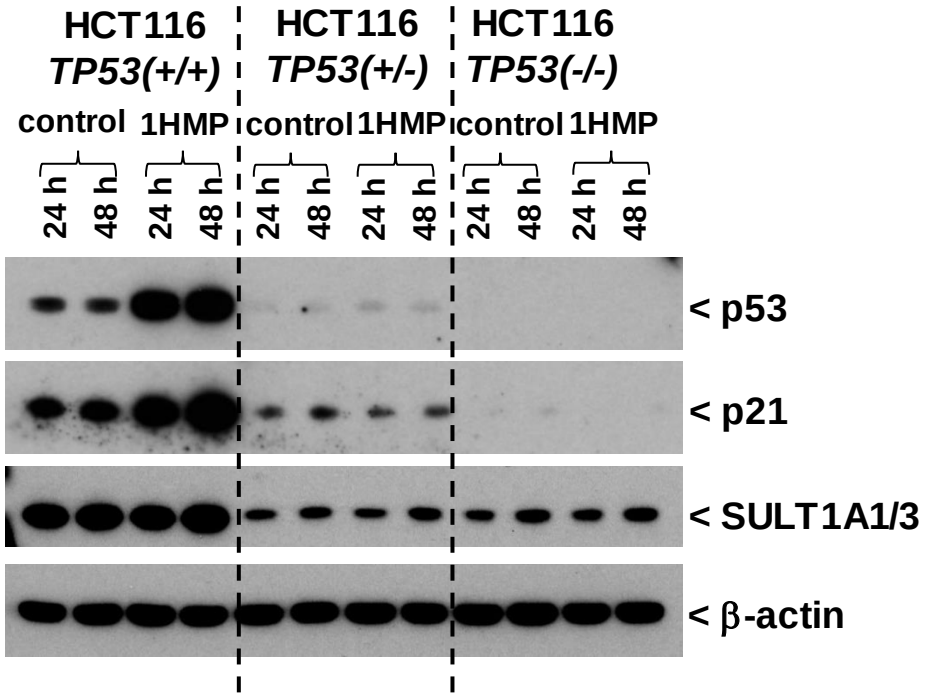

Figure 4